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Both developing zebrafish and medaka are exquisitely sensitive to the cardiovascular toxicity of all-trans retinoic acid (RA) between fertilization and the onset of blood circulation, with incubation medium concentrations of 3-5 ng/ml applied during the blastula stage causing pericardial edema with subsequent circulatory stasis and severe subcutaneous edema and ascites. The sensitivity of both species to RA decreased dramatically during development, with over 40 ng/ml required to cause edema with embryos treated after body pigmentation, and over 300 ng/ml required to cause edema in hatched fry. Zebrafish and medaka embryos are comparably sensitive to the cardiovascular toxicity of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) when treated at fertilization, with 2-3 ng/g TCDD in embryos causing lethality. Thus these species are approximately 40X less sensitive to the cardiovascular toxicity and lethality caused by TCDD exposure early in development than are lake trout embryos in which the LC50 is 0.065 ng/g. Both fish species demonstrate a TCDD cardiovascular toxicity syndrome similar to that seen following RA exposure, with initial pericardial edema followed by circulatory stasis, subcutaneous edema and ascites. Histologic lesions in TCDD-treated zebrafish included ballooning degeneration and necrosis of brain and epithelial tissues such as kidney, pancreas and liver. Molecular components of basement membrane of cardiovascular and epithelial tissues, including fibronectin, collagen IV and laminin, have been revealed in frozen sections of zebrafish and medaka using immunoperoxidase or immunofluorescence detection methods.

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CARDIOVASCULAR TOXICITY OF ENVIRONMENTAL CONTAMINANTS TO DEVELOPING FISH--MOLECULAR MECHANISMS

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Materials and Methods

Broodstock Maintenance

Medaka and zebrafish broodstock were obtained from Carolina Biological Laboratories and were immersed for ½ hr in water containing 150 ppm formalin to reduce parasite burdens. Fish were housed in 40-l glass aquaria receiving flowing dechlorinated tap water at 26-27C. Approximately 6 males and 6 females of each species were housed per tank. The photoperiod was held at 14-h light/10-h dark. Fish were fed flake tropical fish food (TetraMin, Tetrawerke, W. Germany) and brine shrimp (Argent Chemical Laboratories, Redmond, WA) ad libitum once daily.

Egg Collection and Embryo Rearing

Medaka eggs were collected by netting egg-bearing females and gently rubbing eggs onto the net. Eggs were rinsed in distilled water and placed in a plastic petri dish containing embryo rearing solution (Kirchen and West, 1976). Fine forceps were used to remove adhesive filaments from the medaka eggs. Zebrafish eggs were collected in one of two ways. In the first method, 3 females and 3 males were placed for up to an hour in a 500 ml plastic tub floated in a fish tank. A nylon net placed in the tub separated the fish from their eggs. In the second method, 40-l aquaria containing 6 males and 6 females were equipped with a plastic undergravel filter, with no gravel added to the tank. The circulator pump for the undergravel filter was used to lift eggs from beneath the plastic "false bottom" formed by the undergravel filter. Eggs were rinsed in distilled water. Eggs of each species were examined under a stereomicroscope and unfertilized eggs were discarded. Ten fertilized eggs of either species were placed into glass scintillation vials containing 10 ml of embryo rearing solution and were incubated in a low temperature incubator at 25 C.

Toxicant Exposures

All-Trans Retinoic Acid

All-trans retinoic acid was obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions were prepared in HPLC grade acetone in foil covered glass vials. Stock solutions were stored at -20C. Final dosing solutions were prepared in embryo rearing solution and were added to scintillation vials immediately before adding embryos or fry. Acetone concentrations in the dosing solutions were 1/1000 or less. For each toxicant dose, or acetone vehicle control groups, 2 vials each containing 10 embryos in 10 ml of dosing solution were prepared. Embryos were examined daily throughout development using a Wild M20 stereomicroscope or a Wild macroscope.

TCDD

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) exposures were conducted at the University

of Wisconsin, Madison, by Dr. Richard Peterson and Dr. Tala Henry. Tritiated [1,6-H] TCDD was obtained from Chemsyn Science (Lenexa, KA) and purified to final radiochemical purity of >99% by reverse-phase high performance liquid chromatography as described by Olson (1986). HPLC grade acetone was used as the carrier for TCDD. Freshly fertilized zebrafish eggs were exposed to graded doses of aqueous TCDD for 1 hour and were then sampled for residue analysis or placed in fresh rearing solution for toxicity and mortality studies. Egg TCDD residues were determined using scintillation counting as described by Spitsbergen et al (1991). LD50 (lethal dose to 50% of animals) and ED50 (dose causing lesions in 50% of animals) concentrations for yolk sac and pericardial edema were determined with Probit Analysis using SAS statistical software.

Assessment of Molecular Components of Extracellular Matrix of Cardiovascular Tissues

Components of extracellular matrix were visualized using immunoperoxidase techniques applied to paraffin-embedded, glycol methacrylate-embedded or frozen sections of fish tissues. Immunofluorescence techniques were also applied to frozen and glycol methacrylate-embedded tissues. Details for these methods are reported in Appendices 1-4. Primary antibodies assessed for reactivity to fish tissues are listed in Appendix 5.

Preparation of Zebrafish Fry from TCDD Toxicity Study for Histologic Examination

Fry were dehydrated in a graded series of ethanol solutions, embedded in glycol methacrylate, and step sections 4 um thick were prepared using glass knives on a Reichert-Jung 1150/Autocut microtome. Sections were stained with Hematoxylin and Eosin.

Results

Molecular Components of Extracellular Matrix of Fish

Table 1 summarizes results of studies investigating the reactivity with fish tissue of various commercial antibodies produced by immunization of laboratory mammals with mammalian antigens. Results of immunoperoxidase and immunofluorescence assays were considered positive if specific staining of basement membrane of vascular and epithelial tissues was clearly much stronger than background staining on test slides and on negative control slides incubated with nonimmune serum instead of primary test antibodies.

Stage-Specific Dose-Response Studies of All-Trans Retinoic Acid Cardiovascular Toxicity in Developing Zebrafish and Medaka

These studies verified that at early time points in development, prior to or during cardiac morphogenesis, low concentrations of all-trans retinoic acid (RA) caused truncation of the cardiac tube, with development of a single chamber (atrium) in medaka, as had been previously reported in zebrafish (Stainier and Fishman, 1992). In medaka, exposure of embryos in stage 10-11 (high

blastula) or earlier to 5 ng/ml or greater concentrations of RA resulted in truncation of the cardiac tube. In both species, at doses below, or developmental stages beyond those at which gross cardiac malformations occurred, a syndrome of cardiovascular toxicity occurred following treatment with RA. When treatment of developing zebrafish or medaka occurred prior to hatching, the RA toxicity syndrome began with pericardial edema, which increased in severity and was followed by complete circulatory stasis, diffuse subcutaneous edema and ascites. Sensitivity to RA-induced cardiovascular dysfunction decreased throughout development in both species, such that following hatch, fry of both species required at least 100X more RA in the incubation medium in order to induce cardiovascular dysfunction. Following hatch, both species became relatively refractory to pericardial edema, but still developed severe diffuse subcutaneous edema and ascites following relatively high doses of RA.

Toxicity of TCDD to Zebrafish Embryos

Negligible mortality occurred in TCDD-treated eggs prior to hatch, with mortality in sham and vehicle control groups as well as TCDD-treated groups remaining below 2%. Hatching began at 36 and was completed by 96 hours post-fertilization (HPF). Pericardial edema developed in treatment groups which contained greater than 1.5 ng/g TCDD. Pericardial edema developed by 60 hours post-treatment, which was also 60 HPF. TCDD-treated fry with pericardial edema developed subcutaneous edema of the yolk sac and body, as well as ascites. The subcutaneous edema and ascites in TCDD-treated fry arose within 1 to 3 days following pericardial edema and became progressively more severe, finally culminating in complete circulatory stasis and ultimately death. Mortality began in TCDD treatment groups at 100 HPF and reached 100% in the highest dose groups by 240 HPF. The LD50 and 95% fiducial limits (10 days post-treatment) for concentrations of TCDD in zebrafish eggs exposed to aqueous TCDD at fertilization was 2.45 (1.94-2.89) ng/g TCDD. The ED50s for pericardial edema and yolk sac edema were 2.16 and 2.43 ng/g, respectively. Foreshortened maxillae were observed in the skulls of 100% (5 of 5) fry in each of the 4.58 and 7.88 ng/g treatment groups examined at 104 HPF. At 128 HPF 4 of 6 and 5 of 5 fry in the 3.6 and 8.58 ng/g treatment groups, respectively, showed foreshortened maxillae.

Table 1

Reactivity of Fish Tissues With Primary Antibodies Against Extracellular Matrix Using Immunoperoxidase Assays[1]

Tissue Antigen Evaluated	Primary Antibody (Dilution)	Species Tested	Tissue Tested	Section Type	Fixation[2]	Antigen Retrieval	Chromogen[3]	Results[4]
Collagen IV	M785 (1:20)	Bluegill (Adult)	Rete of Gas Bladder	Paraffin	NBF	Trypsin	DAB	Neg
Collagen IV	M785 (1:20)	Medaka (Adult)	Saggital Section	Paraffin	NBF	Pronase	AEC	Neg
Collagen IV	AB748 1:1000	Medaka (Adult)	Saggital Section	Frozen	Acetone	None	AEC	S Pos
Laminin	4C7 (1:20)	Bluegill	Rete of Gas Bladder	Paraffin	NBF	Pronase	AEC	Neg

[1] Positive control tissues were dog kidney (paraffin and frozen sections), mouse kidney (GMA and frozen sections) and human tonsil (paraffin sections). All showed strong positive reactions with basement membrane of vascular tissues and epithelium using each of the primary antibodies. Negative controls included mammalian and fish tissues treated with nonimmune mouse ascites (for monoclonal test antibodies) or nonimmune rabbit serum (for polyclonal test antibodies) instead of primary test antibody.

[2] NBF=neutral buffered formalin

[3] DAB=diaminobenzidine; AEC=aminoethylcarbozole

[4] S Pos=strong positive; W Pos=weak positive; Neg=negative

Table 1, Continued

Tissue Antigen Evaluated	Primary Antibody (Dilution)	Species Tested	Tissue Tested	Section Type	Fixation[5]	Antigen Retrieval	Chromogen[6]	Results[7]
Laminin	L9393 (1:50)	Medaka	Saggital Section	Paraffin	NBF	Microwave, then Pronase	AEC	Neg
Laminin	L9393 (1:100)	Zebrafish Fry[8]	Saggital Section	GMA	ZNF	Pronase	AEC	Neg
Laminin	L9393 (1:100)	Medaka (Adult)	Heart	GMA	Unfixed, Lyophilized	None	DAB	Neg
Laminin	AB949 (1:400)	Medaka (Adult)	Saggital Section	Frozen	Acetone	None	AEC	S Pos
Fibronectin	F3648 (1:50)	Bluegill	Rete	Paraffin	NBF	Microwave, Pronase	AEC	Neg

[5] NBF=neutral buffered formalin; ZNF=zinc formalin (Dapson, 1993)

[6] DAB=diaminobenzidine; AEC=aminoethylcarbozole

[7] S Pos=strong positive; W Pos=weak positive; Neg=negative

[8] Freshly hatched.

Table 1, Continued

Tissue Antigen Evaluated	Primary Antibody (Dilution)	Species Tested	Tissue Tested	Section Type	Fixation[9]	Antigen Retrieval	Chromogen[10]	Results[11]
Fibronectin	F3648 (1:100)	Zebrafish Fry	Sagital Section	GMA	ZNF	Pronase	AEC	Neg
Fibronectin	AB 1946 1:1000	Medaka (Adult)	Sagital Section	Frozen	Acetone	None	AEC	S Pos
Heparan sulfate	MAB 459472 (1:20)	Bluegill	Rete	Paraffin	NBF	Pronase	AEC	Neg
Heparan sulfate	MAB 45947 (1:20)	Medaka (Adult)	Sagital Section	Frozen	Acetone	None	DAB	Neg

[9] NBF=neutral buffered formalin; ZNF=zinc formalin (Dapson, 1993)

[10] DAB=diaminobenzidine; AEC=aminoethylcarbozole

[11] S Pos=strong positive; W Pos=weak positive; Neg=negative

Table 2

Reactivity of Fish Tissues With Primary Antibodies Against Extracellular Matrix Using Immunofluorescence Assays[12]

Tissue Antigen Evaluated	Primary Antibody (Dilution)	Species Tested	Tissue Tested	Section Type	Fixation[13]	Antigen Retrieval	Antigen Detection	Results[14]
Collagen IV	COL-94 (1:200)	Zebrafish Fry[15]	Saggital Section	Frozen	Acetone	None	FITC	Neg
Collagen IV	COL-94 (1:200)	Medaka (Adult)	Saggital Section	Frozen	Acetone	None	FITC	Neg
Collagen IV	AB748 1:2000	Medaka (Adult)	Saggital Section	Frozen	Acetone	None	FITC	S Pos
Collagen IV	AB748 (1/400)	Medaka	Heart	GMA	Unfixed, Lyophilized	None	FITC	S Pos

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[12] Positive control tissues were dog kidney, mouse liver and kidney (frozen and GMA sections).. All showed strong positive reactions with basement membrane of vascular tissues and epithelium using each of the primary antibodies. Negative controls included mammalian and fish tissues treated with nonimmune mouse ascites (for monoclonal test antibodies) or nonimmune rabbit serum (for polyclonal test antibodies) instead of primary test antibody.

[13] NBF=neutral buffered formalin; ZNF=zinc formalin (Dapson, 1993)

[14] S Pos=strong positive; W Pos=weak positive; Neg=negative

[15] Freshly hatched.

Tissue Antigen Evaluated	Primary Antibody (Dilution)	Species Tested	Tissue Tested	Section Type	Fixation[16]	Antigen Retrieval	Antigen Detection	Results[1]
Laminin	L9393 (1:200)	Zebrafish Fry	Sagittal Section	Frozen	Acetone	None	FITC	S Pos
Laminin	L9393 (1:200)	Medaka (Adult)	Sagittal Section	Frozen	Acetone	None	FITC	S Pos
Laminin	AB949 (1:250)	Medaka (Adult)	Sagittal Section	Frozen	Acetone	None	FITC	Neg
Fibronectin	F3648 (1:200)	Zebrafish Fry	Sagittal Section	Frozen	Acetone	None	FITC	S Pos
Fibronectin	F3648 (1:200)	Medaka (Adult)	Sagittal Section	Frozen	Acetone	None	FITC	S Pos
Fibronectin	AB 1946 (1:250)	Medaka (Adult)	Sagittal Section	Frozen	Acetone	None	FITC	S Pos
Fibronectin	AB 1946 (1:400)	Medaka (Adult)	Sagittal Section	GMA	Unfixed, lyophilized	None	FITC	S Pos

[16] NBF=neutral buffered formalin; ZNF=zinc formalin (Dapson, 1993)

[17] S Pos=strong positive; W Pos=weak positive; Neg=negative

Table 3

Stage-Specific Cardiovascular Toxicity of All-Trans Retinoic Acid in Developing Zebrafish

Stage of Devel-opment[18]	**Hours Post-Fertil-ization (25 C)**	**Dose Range (ng/ml)**	**MDE**[19]	**Time to Edema (Days)**	**MDS**[20]	**Time to Circul-atory Stasis (Days)**	**Edema in 100%**[21]	**Circul-atory Stasis in 100%**[22]
Blastula	4-6	0.5-10	1.0	2-5	1.0-3.0	2-5	2.0	2.0[23]
20-Somite	24	2-80	10	2-5	10	2-5	20	20
Prim-25	48	20-200	20	2-4	20	2-4	40	40
Long-pec	72	40-100	40	2-3	40	2-5	100	100
Pro-truding Mouth	96	20-640	160	2-3	160	2-3	320	640

[18] Developmental stages described by Kimmel et al. (1995)

[19] Minimum dose causing edema.

[20] Minimum dose causing circulatory stasis.

[21] Dose causing edema in 100% of treated individuals.

[22] Dose causing circulatory stasis in 100% of treated individuals.

[23] At medium concentrations of 3 ng/ml or greater applied at early blastula stage, circulation never established.

Table 4

Stage-Specific Cardiovascular Toxicity of All-Trans Retinoic Acid in Developing Medaka

Stage of Devel-opment[24]	Hours Post-Fertil-ization (25 C)	Dose Range (ng/ml)	MDE[25]	Time to Edema (Days)	MDS[26]	Time to Circul-atory Stasis (Days)	Edema in 100%[27]	Circul-atory Stasis in 100%[28]
10-11; High Blastula	6-9	0.5-20	1.0	2-5	2.0	3-5	5.0	5.0[29]
17; Neurula	24	5-100	10	2-5	10	3-5	20	20[30]
23; Heart-beat	48	5-100	15	2-5	15	3-5	30	30
27; Pectoral Fin Bud	72	10-200	20	2-3	40	2-3	80	120
29	96			3-5		3-5	300	300

[24] Developmental stages described by Kirchen and West (1974).

[25] Minimum dose causing edema.

[26] Minimum dose causing circulatory stasis.

[27] Dose causing edema in 100% of treated individuals.

[28] Dose causing circulatory stasis in 100% of treated individuals.

[29] Vitelline vessels and circulation failed to develop in all embryos treated with 5 ng/ml or more retinoic acid at high blastula stage. At 3 ng/ml, 30% of embryos lacked vitelline veins and circulation.

[30] Circulation never established at doses of 15 ng/ml or greater.

Table 4, Continued

Stage-Specific Cardiovascular Toxicity of All-Trans Retinoic Acid in Developing Medaka

Stage of Development[31]	Hours Post-Fertilization (25 C)	Dose Range (ng/ml)	MDE[32]	Time to Edema (Days)	MDS[33]	Time to Circulatory Stasis (Days)	Edema in 100%[34]	Circulatory Stasis in 100%[35]
31	120			5-6		5-7	3,000	3,000
35	200		5,000					
Post-Hatch	336		>5,000					

[31] Developmental stages described by Kirchen and West (1974).

[32] Minimum dose causing edema.

[33] Minimum dose causing circulatory stasis.

[34] Dose causing edema in 100% of treated individuals.

[35] Dose causing circulatory stasis in 100% of treated individuals.

Histologic Lesions in TCDD-Treated Zebrafish Embryos

Appendices 6 and 7 summarize the microscopic lesions observed in zebrafish fry exposed as fertilized eggs to graded doses of TCDD. At 104 hours post-fertilization, lesions were most marked in the 7.88 ng/g treatment group (Vial 8). Two of 4 of these fish examined had severe pericardial edema histologically, one having pericardial hemorrhage also. These two fish had mild ballooning degeneration and necrosis of the brain. These 2 fish also had uninflated, underdeveloped gas bladders. At 104 hours post-fertilization, normal control fish had a characteristic layer of eosinophilic extracellular matrix between the inner epithelium and the smooth muscle layer of the gas bladder. Fish with underdeveloped, uninflated gas bladders lacked this layer. The two severely affected fish also showed subcutaneous edema of the head and yolk sac, with one of the fish showing mild, locally extensive endomysial edema and myocyte necrosis in skeletal muscle on the anterior trunk. The gills of the 7.88 ng/g treatment group were relatively underdeveloped compared to controls, having less skeletal muscle supporting the base of gill arches and having less growth of the cartilage core of the gill arch. Glycogen depletion from hepatocytes was not yet evident at 104 hr post-fertilization, even in the 7.88 ng/g treatment group. Despite the presence of pericardial edema in the 4.58 ng/g treatment group, neither epithelial nor brain lesions were observed in these fish at this time point. Only mild locally extensive endomysial edema and myocyte necrosis were observed on the anterior trunk in 1 of 5 fish in the 4.58 ng/g group at 104 hr post-fertilization. Mild pericardial edema was observed histologically in 1 of 5 sham control fish at 104 hours post-fertilization. This fish also had an uninflated, underdeveloped gas bladder. These cardiovascular lesions, and associated developmental abnormalities, in the sham control fish most likely resulted from handling trauma. One of 5 vehicle control fish at 104 hr post-fertilization also had mild locally extensive myocyte necrosis in the trunk skeletal muscle. This lesion is most likely due to a localized vascular disturbance caused by trauma. The TCDD treatment groups showing foreshortened maxillae at 104 hr post-fertilization showed less growth of skeletal muscle and cartilage in the head when compared to control groups.

More severe epithelial and brain lesions were observed in the 3.6 and 8.58 ng/g treatment groups at 128 hr post-fertilization than in 7.88 and 4.58 ng/g treatment groups at 104 hr post-fertilization. In the 3.6 ng/g group, 1 of 5 brains examined showed severe ballooning degeneration and necrosis of neuroepithelium throughout all major brain regions, 1 of 5 showed moderate and 2 of 5 showed mild ballooning degeneration and necrosis in the brain. Ballooning degeneration and/or necrosis was observed in various epithelial tissues in the 3.6 ng/g group, including renal tubular epithelium of 3 of 6 fish, hepatocytes of 1 of 6 fish, endocrine pancreas of 2 of 6 and exocrine pancreas of 1 of 6 fish. Moderate to severe pericardial edema was observed in 5 of 6 fry in the 3.6 ng/g group, and pericardial hemorrhage occurred in 1 of these fish. These fish with pericardial edema also showed glycogen depletion from hepatocyte cytoplasm, round cytoplasmic vacuoles in hepatocytes and arrested development of the gill. In the 3/6 ng/g group, only the fish without histologic evidence of pericardial edema showed formation of filaments on the gill arches. The remaining 5 of 6 fish with underdeveloped gills also showed less skeletal muscle and less growth of cartilage in the gill arches compared to control groups at this time

point. The 5 fish with pericardial edema also had underdeveloped, uninflated gas bladders. At this time point, control fish with inflated gas bladders showed expansion of the connective tissue of the wall of the gas bladder, with disappearance of the eosinophilic layer of extracellular matrix which was present at 104 hr post-fertilization. All 5 fish with underdeveloped, uninflated gas bladders at 128 hr post-fertilization lacked expansion of the connective tissue of the gas bladder wall and 1 showed the eosinophilic extracellular matrix layer characteristic of control gas bladders at 104 hr post-fertilization. Four of 5 fish with pericardial edema showed subcutaneous edema of the head, trunk and yolk sac. Four of 6 of the 3.6 ng/g fry examined at 128 hr post-fertilization had endomysial edema of the skeletal muscle of the trunk, including the fish without histologic evidence of pericardial edema. One of 5 sham control fry examined at 128 hr post-fertilization had mild pericardial edema, depletion of glycogen from liver hepatocytes and mild ballooning degeneration of neuroepithelial cells of the brain. These lesions in the control fish presumably resulted from trauma.

Discussion

Molecular Components of Basement Membrane of Tissues of Zebrafish and Medaka

Visualization of molecular components of basement membrane of zebrafish and medaka using commercially available antibodies and immunoperoxidase or immunofluorescence methods proved more challenging than expected. We did not achieve success in demonstrating any of the antigens evaluated, including collagen IV, fibronectin, laminin and heparan sulfate in formalin or zinc formalin-fixed fish tissue, although the antibodies and methods evaluated worked well with mammalian positive control tissues. Neither enzymatic (trypsin or pronase) nor microwave techniques for antigen retrieval were effective with aldehyde-fixed fish tissues. Strong positive staining of basement membrane in fish tissues with antibodies to collagen IV, fibronectin and laminin occurred only in frozen, acetone-fixed sections or unfixed, lyophilized GMA-embedded sections. Because optimization of these immunochemical techniques applied to fish tissues took longer than anticipated, evaluation of basement membrane components at various developmental stages of medaka and zebrafish was not possible, either in control fish or in toxicant-treated fish. However, the methods developed for assessing components of fish basement membrane should be valuable in future toxicology or neoplasia studies in fish.

Cardiovascular Toxicity of All-Trans Retinoic Acid (RA) in Developing Zebrafish and Medaka

Abnormal cardiovascular function in developing zebrafish or medaka treated with retinoic acid prior to or during the 9-somite stage occurs at least in part because of gross malformation of the developing cardiac tube, with deletion of part or all of the bulbus arteriosus and/or the ventricle (Stainier and Fishman, 1992). The mechanisms underlying the cardiovascular toxicity syndrome occurring with later exposure of these embryos to RA are more subtle and remain uncertain. Due to early termination of this research project, molecular components of cardiovascular tissues of toxicant-treated fish were not assessed, nor were physiological

parameters of cardiovascular function or inflammatory mediators in cardiovascular tissues. The specific developmental events which correspond to the progressive decrease in susceptibility of cardiovascular tissues to adverse effects of RA during development remain to be clarified.

Toxicity and Pathologic Lesions in TCDD-Treated Zebrafish Early Life Stages

Zebrafish and medaka embryos (Wisk and Cooper, 1990) are comparably sensitive to the cardiovascular toxicity of TCDD when treated as fertilized eggs, with 2-3 ng/g TCDD in embryos causing lethality. Persistence of TCDD in yolk lipid results in exposure of fish embryos to TCDD throughout embryonic development, when treatment occurs at fertilization (Spitsbergen et al, 1991). In contrast to RA, which alters patterning of heart chambers in the developing cardiac tube in zebrafish and medaka when treatment occurs early in embryonic development, treatment of zebrafish or medaka embryos with TCDD early in development does not result in grossly observable malformations of the cardiac chambers. The TCDD-induced cardiovascular toxicity syndrome is expressed relatively late in development, well after cardiac chamber formation, cardiac looping and establishment of circulation in the major vascular beds.

Several differences are evident between the TCDD toxicity syndrome in developing lake trout or medaka and that in zebrafish. In medaka, circulatory derangements and subsequent degeneration in the brain and retina occur well before hatch. In lake trout which contained 400 ppt TCDD, circulatory dysfunction and degeneration of neuroepithelial tissues occurred shortly before and during hatch. In contrast, circulatory derangements, edema and degeneration and necrosis of the brain occur in zebrafish only well after hatch. The congestion and hemorrhage which was prominent in the brain, eye and spinal cord of lake trout early life stages treated with lethal doses of TCDD were not observed in zebrafish fry following treatment of eggs with lethal TCDD doses. The lack of degeneration or necrosis in eye or spinal cord of zebrafish, and the relatively milder lesions in skeletal muscle of lethally intoxicated zebrafish fry compared to lake trout fry may be because of the smaller size of zebrafish, allowing diffusion to supply sufficient oxygen to all but the most metabolically active tissues.

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APPENDIX 1:

PROTOCOL FOR IMMUNOPEROXIDASE STAINING OF TISSUES EMBEDDED IN PARAFFIN[36]

Apparatus/Reagents:

Microprobe immunohistochemistry apparatus (Fisher Scientific, Pittsburgh, PA) utilized with Zymed (South San Francisco, CA) streptavidin-biotin Histostain-SP kit containing broad spectrum reagents reactive to mouse and rabbit primary antibodies.

Deparaffinization:

1. Heat slides at 70 C for 10 min in dry chamber.

2. Place slides into microprobe chamber filled with 3:1 Hemo-DE (Fisher):xylene. Dip slides in this chamber 6X for 1 min, blotting between dips.

3. Perform 5 one min dips with blotting between dips in 100% ethanol.

4. Dip and blot as above in 95% ethanol 4X.

5. Dip in 70% ethanol 1X, blot.

Blocking of Endogenous Peroxidase

1. Dip slides briefly in 0.5% hydrogen peroxide in methanol, blot, then place in this solution 10 min, blot.

2. Dip in 70% ethanol, blot.

3. Dip in phosphate-buffered saline pH 7.4 (PBS) containing 0.4% polyoxyethylene-23 lauryl ether (BRIJ), henceforth called PBS/BRIJ, 4X for 1 min, blotting between dips, blot.

Antigen Retrieval
Enzymatic Antigen Retrieval

1. 0.1% trypsin in PBS at 37 C for 1 hr (for kidney use 0.5% trypsin for 6hr) or 0.5 mg/ml pronase in PBS at 37 C for 1 hr.

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[36] Protocol adapted from that developed by C.A. Smith in the laboratory of Dr. Robert Lewis in the Department of Veterinary Pathology at Cornell University.

APPENDIX 1, CONTINUED

2. Incubate slides with enzyme treatment in humidified chamber.

3. Dip slides in PBS/BRIJ and blot 4X.

Microwave-Assisted Antigen Retrieval

1. Dip slides in distilled water 5 min.

2. Place slides in plastic coplin jars filled with distilled water. Cover loosely with plastic wrap. Place 3 jars in microwave in triangular arrangement to ensure even heating.

3. Heat at 720 watts 5 min. Check water level and again heat at 720 watts 5 min.

4. Remove slides from oven and cool 15 min before continuing.

Blocking of Endogenous Avidin/Biotin

1. Zymed Avidin/Biotin Blocking Kit used.

2. Slides incubated in avidin solution 10 min at room temp.

3. Slides dipped and blotted in PBS 6X.

4. Slides incubated in d-biotin solution 10 min at room temp.

5. Slides dipped and blotted in PBS 6X.

Antibody Incubations

1. Apply blocking serum 10 min at room temp in humidified chamber. Blot.

2. Apply primary antibody and incubate overnight at 4 C in humid chamber. Blot.

3. Dip and blot 6X in PBS/BRIJ.

4. Apply biotinylated secondary antibody and incubate in humid chamber 10 min at room temp.

5. Dip and blot in 6X PBS/BRIJ.

6. Apply diluted streptavidin/peroxidase conjugate 10 min at room temp in humid chamber.

APPENDIX 1, CONTINUED

7. Dip and blot 6X in PBS/BRIJ.

8. Remove slides from Microprobe slide holder.

9. Apply 2 drops of substrate-chromogen mixture. Either diaminobenzidine (DAB) or aminoethylcarbozole (AEC) chromogen used. Incubate in humid dark chamber 5-10 min for AEC and 3 or more min for DAB.

10. Rinse slides well in distilled water.

11. Counterstain in hematoxylin 1-3 min. Rinse in tap water. Place in PBS until blue (approx 30s). Rinse again in distilled water.

12. For AEC, mount with GVA Mount, coverslip. For DAB, dehydrate in graded alcohols, clear in xylene, mount in Permount, coverslip.

APPENDIX 2:

METHOD FOR IMMUNOFLUORESCENCE DETECTION OF ANTIGENS

1. Apply 10% normal goat serum in PBS to slides 10 min at room temp to block nonspecific antibody binding. Aspirate fluid from slides.

2. Cover sections with primary antibody. Incubate 2 hr at room temp in humid chamber.

3. Aspirate fluid and rinse 3X for 10 min with PBS.

4. Apply appropriate dilution (in PBS) of affinity purified goat anti-rabbit or goat anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC)(Zymed Laboratories). Incubate at room temp in humid chamber 30 min.

5. Aspirate fluid and rinse 3X for 10 min with PBS.

6. Mount in glycerin containing 3.4% DABCO (1,4,-diazobicyclo 2,2,2 octane), pH 8.6. Coverslip and examine under epifluorescence with filters for FITC.

APPENDIX 3:

SNAP FREEZING OF TISSUE FOR IMMUNOFLUORESCENCE OR IMMUNOHISTOCHEMISTRY STUDIES[37]

1. Cool isopentane (2-methyl butane) held in a metal pan immersed in liquid nitrogen until opaque droplets form in isopentane.

2. Place tissue in aluminum foil capsule or vial 1/3 full of O.C.T. embedding medium.

3. Holding capsule with metal forceps, plunge into isopentane, but avoid contact of isopentane with tissue. Remove capsule from isopentane within 15s and place in chilled container at -70C or colder.

4. For preparation of frozen sections, mount frozen tissue piece on cryostat chuck using O.C.T. Cut sections 2 um thick using Reichert-Jung cryostat. Place sections on glass slides coated with poly L-lysine (Sigma).

APPENDIX 3A:

LYOPHILIZATION OF UNFIXED TISSUE FOR EMBEDDING IN GLYCOL METHACRYLATE[38]

1. Tissue snap frozen as in Appendix 3. Tissue immediately transferred to freeze plate of Virtis lyophilizer and freeze-dried for 2 days.

2. Tissue transferred to vacuum dessicator, then placed in catalyzed glycol methacrylate. GMA was polymerized in vacuum dessicator at room temperature.

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[37] Method optimized by C.A. Smith in the laboratory of Dr. Robert Lewis of the Department of Veterinary Pathology, Cornell University.

[38] Method modified from Hinton et al, 1988.

APPENDIX 4:

MODIFICATION OF IMMUNOHISTOCHEMISTRY PROTOCOL FOR TISSUE EMBEDDED IN GLYCOL METHACRYLATE[39]

1. Infiltrate tissues with graded series of glycol methacrylate (GMA) monomer, from 50 to 100% diluted with distilled water, avoiding ethanol dehydration.

2. Polymerize GMA at room temperature. Section tissues at 2-4 micrometer intervals and place sections on glass slides coated with Poly L-lysine.

3. Follow the protocol for immunohistochemical staining of paraffin sections in Appendix 1, with the following modifications:

a. Omit deparaffinization.
b. Block endogenous peroxidase for 30 min. with hydrogen peroxide.
c. Retrieve antigen with Pronase for 30 min.

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[39] Method adapted from Hermanns et al, 1986.

APPENDIX 5:

PRIMARY ANTIBODIES TO EXTRACELLULAR MATRIX COMPONENTS TESTED FOR REACTIVITY TO FISH TISSUES

Vendor	**Product Code or #**	**Extracell-ular Matrix Component Used as Immunogen**	**Species Source of Antigen**	**Species in Which Antibody Produced**	**Monoclonal (M) or Polyclonal (P) Antibody**
Chemicon	AB748	Collagen IV	Human	Rabbit	P
Dako	M785	Collagen IV	Human	Mouse	M
Sigma	COL-94	Collagen IV	Human	Mouse	M
Chemicon	AB1946	Fibronectin	Chicken	Rabbit	P
Sigma	F-3648	Fibronectin	Human	Rabbit	P
Chemicon	AB949	Laminin	Human	Rabbit	P
Sigma	L-9393	Laminin	Mouse	Rabbit	P
Dako	4C7	Laminin	Human	Mouse	M
Chemicon	MAB459-472	Heparan sulfate	Bovine	Mouse	M

Appendix 6. Microscopic lesions in zebrafish fry exposed as fertilized eggs to aqueous TCDD

Vial #	Egg TCDD dose (ng/g)	HPF	Microscopic lesions of epithelial tissues: Ballooning degeneration and/or necrosis: Renal tubules	Ballooning degeneration and/or necrosis: Hepatocytes	Ballooning degeneration and/or necrosis: Pancreas	Hepatocytes: Glycogen depletion	Hepatocytes: Round cytoplasmic vacuoles	Arrested development of gill	Microscopic lesions of brain: Ballooning degeneration and/or necrosis
1	0(vehicle)	104	0/5	0/5	0/5	0/5	0/5	0/5	0/5
3	0(sham)	104	0/5	0/5	0/5	0/5	0/5	0/5	0/5
13	4.58	104	0/5	0/5	0/5	0/5	0/5	0/5	0/5
8	7.88	104	0/4	0/4	0/4	0/4	0/4	4/4	2/4 (1)[2]
5	0(sham)	128	0/5	0/5	0/5	1/5	0/5	0/5	1/5(1)
2	0(vehicle)	128	0/5	0/5	0/5	0/5	0/5	0/5	0/5
16	0.83	128	0/4	0/4	0/4	0/4	0/4	0/4	0/4
11	3.60	128	3/6	1/6	2/6	5/6	5/6	5/6	5/5(1-3)
6	8.58	128	0/5	2/5	0/5	5/5	5/5	5/5	2/5 (1-2)

[1]Hours post-fertilization

[2]1: mild lesion
2: moderate lesion
3: severe lesion

Appendix 7. Microscopic lesions in zebrafish fry exposed as fertilized eggs to aqueous TCDD

			Microscopic lesions of mesenchymal tissues								
			Edema of subcutis			Heart		Skeletal muscle		Gas bladder	
Vial #	Egg TCDD Dose (ng/g)	HPF[1]	Head	Trunk	Yolk sac	PCE[2]	PCHM[3]	Endomysial edema	Myocyte necrosis	Under-developed	Uninflated
1	0(vehicle)	104	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
3	0(sham)	104	0/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	1/5
13	4.58	104	0/5	0/5	0/5	3/5	0/5	1/5	1/5	0/5	0/5
8	7.88	104	2/4	0/4	2/4	3/4	1/4	1/4	1/4	2/4	2/4
5	0(sham)	128	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
2	0(vehicle)	128	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
16	0.83	128	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
11	3.60	128	4/6	4/6	4/6	5/6	1/6	4/6	0/6	5/6	5/6
6	8.58	128	5/5	5/5	5/5	5/5	2/5	5/5	0/5	5/5	5/5

[1]Hours post-fertilization

[2]Pericardial edema

[3]Pericardial hemorrhage